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TITLE: Validation of a Pre-Clinical Model for the Investigation of Menarcheal Age on Breast Cancer Risk

PRINCIPAL INVESTIGATOR: Pepper J. Schedin, Ph.D.

CONTRACTING ORGANIZATION: University of Colorado Health Sciences Center
Aurora, Colorado 80045-0508

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Pepper J. Schedin, Ph.D.

E-Mail: pepper.schedin@uchsc.edu

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Progress Report; W81XWH-05-1-0499

Title: Validation of a Pre-Clinical Model for the Investigation of Menarcheal Age on Breast Cancer Risk

Beginning Date 09-01-2005, with a one year extension granted for an ending date of 08-31-2007. A one year extension was granted because the PI, Dr. Pepper Schedin, moved her lab from AMC Cancer Research Center in Denver, CO to the University of Colorado Health Sciences Center. This move resulted in a 6 month delay in finalizing the grant award. As a result, work on this grant was delayed until June 2006 and thus this final report represents work accomplished over an 18 month period from June 2006-January 2008.

Body: Describes the research accomplishments associated with each task in the approved statement of work.

Tasks associated with Aim 1 were completed and reported in the first grant report of 12/07. These accomplished tasks, as written in the first grant report, are included below.

Task 1a. Identify Sprague-Dawley female rats with early and late onset of sexual maturation.

Months 1-2. Rationale: As with humans, outbred Sprague Dawley (SD) female rats demonstrate a bell-shaped curve relationship between age and maturation of the ovaries. Thus, rats can be segregated into groups of early and late onset of sexual maturation. Vaginal opening was used as the marker for onset of menarche because VO is an estrogen dependent process, thus is dependent on ovarian function. Most rats have one complete estrous cycle within10 days of VO. Two hundred 21 day of age (p21) female SD rats (with precise date of birth known) were fed AIN-93G diet, a defined diet optimized for rapidly growing young rats. Starting at p26, the rats were evaluated daily for onset of vaginal opening (VO), by visual inspection.

Status: Completed.

Results: Two hundred 20 day of age female SD rats (p21) were obtained from Sprague-Dawley Harlan and monitored daily by visual inspection for vaginal opening. Thirty nine rats had vaginal opening between 28 and 30 days of age, 123 had vaginal opening between 31and 33 days of age and 38 had vaginal opening at 34 days of age or later. The distribution of age at vaginal opening is shown in **Figure 1**. For this study, we utilized rats with vaginal opening at 30 days of age or younger for the early VO group and those with vaginal opening of 34 days of age or later as the late VO group.

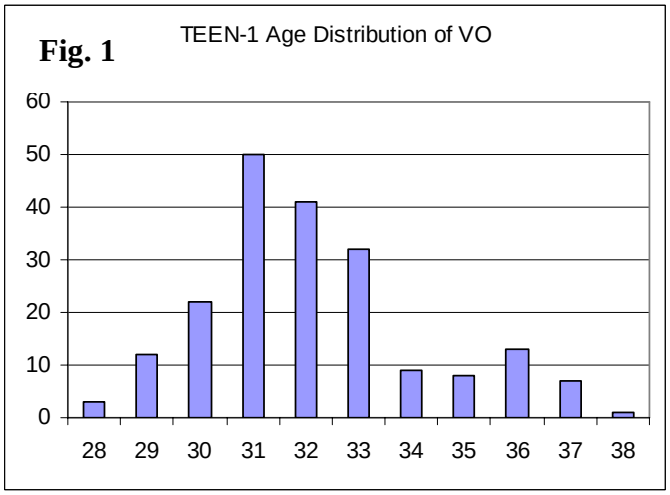

Fig. 1 Distribution of age at vaginal opening (VO) in 200 female SD rats. Rats with VO of 30 days and younger were assigned to the early VO group and rats with a VO of 34 days of age and later to the late VO group.

Unexpected Problems Encountered: Based on previous studies, we had anticipated obtaining a wider spread in age at VO than was found in this current study. One reason for the discrepancy may be that previous studies were performed using non-ventilated cages whereas this current study was performed using ventilated cages.

We have discovered significance differences in estrous cycling and breeding habits between rats in the two different caging environs, thus ventilated caging may have influenced age at VO. As a result, we had significantly fewer rats in the early VO and late VO groups than anticipated (39 and 38 per group compared to an expected 50 per group). Thus, we decided to focus our efforts on Aim 2 of the grant, as we did not have enough animals to carry out the carcinogenesis study proposed in Aim 1. **This information was provided in the grant report submitted 12/07.**

Task 1b. Determine if there is a relationship between 1) body weight and age at VO and 2) age at VO and adult body weight. Rationale: In humans, correlations exist between body mass index in childhood and age of sexual maturation and adult body weight, however body mass index alone does not account for early onset of puberty. Starting at p21, body weights were taken twice weekly to study end to characterize these relationships in the rat. **Status: Completed.**

Results: The relationship between body weight and onset of sexual maturation observed was complex. In **Figure 2**, it can be seen that overall, the rats that had early VO were as a group heavier than the rats that had late VO. This data models the relationship observed between onset of puberty and body weight in girls. Of note, and as can be seen in **Figure 2**, the difference in body weight between groups was observed early, by 28 days of age, and persisted with age but did not increase in magnitude with time. **This information was provided in the grant report submitted 12/07**.

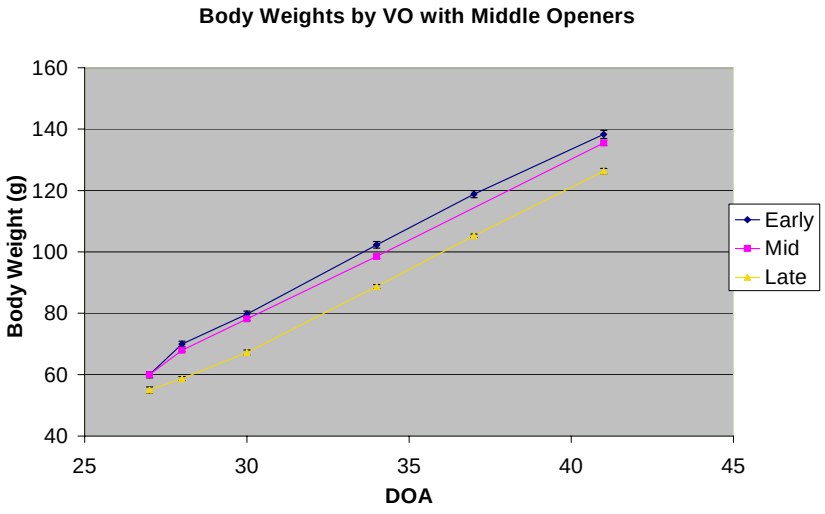

Fig. 2 Rats with early VO had, as a group, higher body weights than rats with late VO.

In **Figure 3**, the weight of each rat on the day of VO is shown. It can be seen that overall, animals in the early VO group reached sexual maturation at a much lower body weight than rats in the late VO group. These data suggest that body weight alone does not account for entry into VO, but rather a combination of body weight in conjunction with a 'permissive' endocrine status/profile is likely to be responsible for onset of VO.

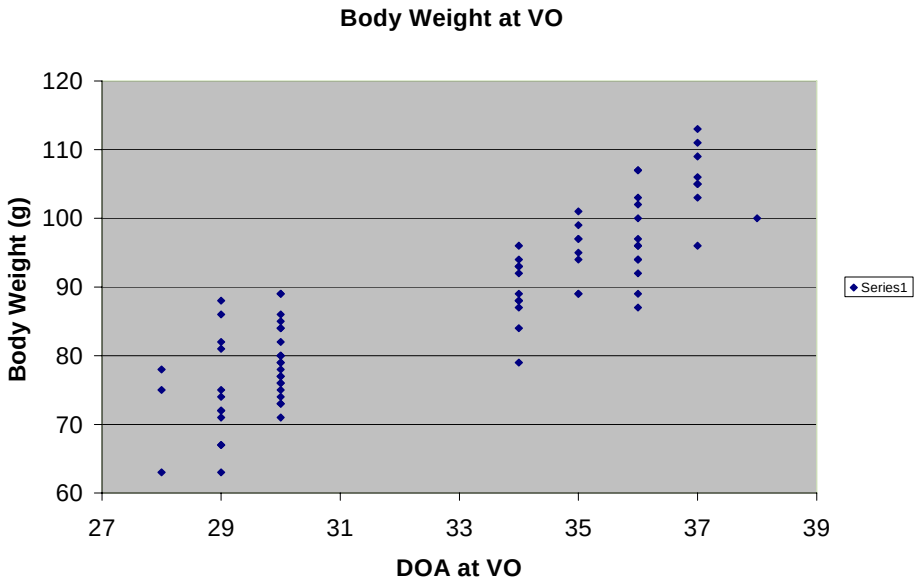

Fig. 3 Body weight at time of VO was lower in the early VO group than in the late VO group.

Task 2. Determine whether age at VO alters circulating 17-β-estradiol, progesterone and IGF-1 levels in the mature rat. Months 3-5.

Task 2a. Cycle Regularity. At p63, 25 rats per group will be evaluated daily for stage of estrous by cervical lavage. The question of whether age at VO influences the length or regularity of the cycles will be determined over a two week period.
Status: Complete
Results: No differences in cycle length, regularity or days in follicular phase (Estrus stage) were observed between early and late VO groups. To

Task 2a2. Blood collection for hormone analyses. To control for variation in circulating hormone levels that could be due to either length of cycle or irregularity of cycle, only rats identified to have regular four day cycles were selected for hormone evaluation. One milliliter of blood per rat was obtained by orbital eye bleed at two stages of the estrous cycle; correspond to low and high circulating ovarian hormone levels, respectively. Circulating hormone levels are lowest during diestrus 1 of the cycle and blood samples for this stage were collected at 12:00 pm. Conversely, blood was collected at 6:00 pm on day of proestrus, a stage and time of day that corresponds to the highest levels of estrogen and progesterone, as detection by RIA analyses.
Status: Animal Work Completed. We have obtained blood from regular cycling rats (all 5 day cyclers) during proestrus and diestrus 1 stages of their cycle in 14 rats with early VO and 12 rats with late VO.

Task 2b. ELISA analyses for estrogen, progesterone and IGF-1 levels. Immediately after collection, blood samples were centrifuged at 1,200g for 10 min at 4°C and serum stored at -80°C until analyses. For estrogen, progesterone and IGF-1 level determination, each sample will be evaluated in triplicate using commercially available ELISA kits, and levels quantified by comparison with known standards (Alpha Diagnostics, San Antonio, TX). Data will be evaluated using RIAAID software (Hazelton, WA).
Status: Partially completed.

Results: Estradiol levels were determined for 8 rats from the early VO group and 9 rats from the late VO group. As expected, circulating estradiol levels were found to be higher during proestrus than during diestrus 1, regardless of age at VO (**Fig 4**). Inter-animal variation in circulating estradiol levels in the early VO group were less than that observed in the late VO group. Further, there was a trend towards el.evatled levels of E2 is the late VO grpup compared to the early VO group. Significant differences in circulating estradiol levels between groups were not significantly different. These data suggest that early VO does not result in an upregulation of estradiol synthesis that persists into adulthood, as hypothesized.

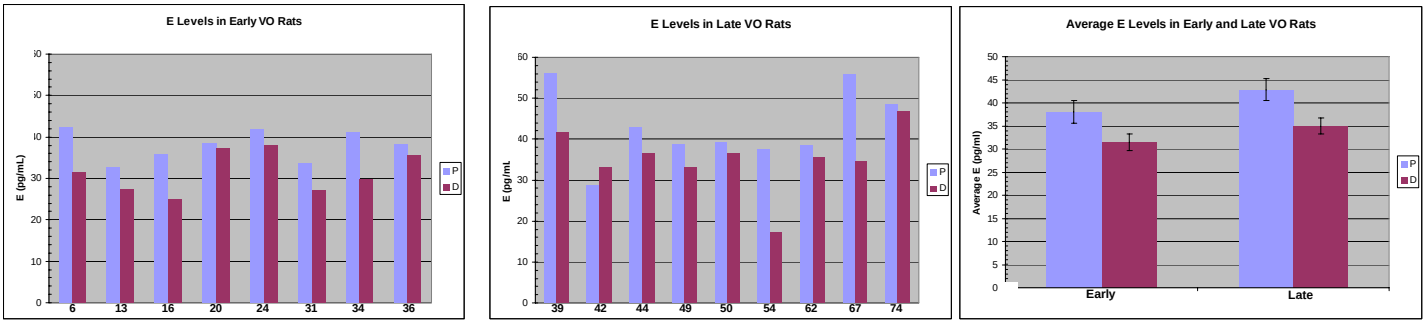

Fig 4 Circulating estradiol levels during proestrus (P) and diestrus 1(D) from individual rats with early (left panel) and late VO (middle panel) and average estradiol levels per group (**left panel**).

Task 3 hormone analyses-new task. We have also collected blood from animals assigned to Task 3 (described below). For this study, blood was collected in the ***estrus phase*** of the cycle from 13 rats with early VO and 11 rats with late VO, and processed for sera and plasma. To date, these samples have been evaluated for estradiol by RIA. To our surprise, circulating estradiol levels were found to be slightly higher in the rats with late VO compared to rats with early VO. **Figure 5** shows the results for individual animals and **figure 6** shows the average circulating estradiol levels per group.

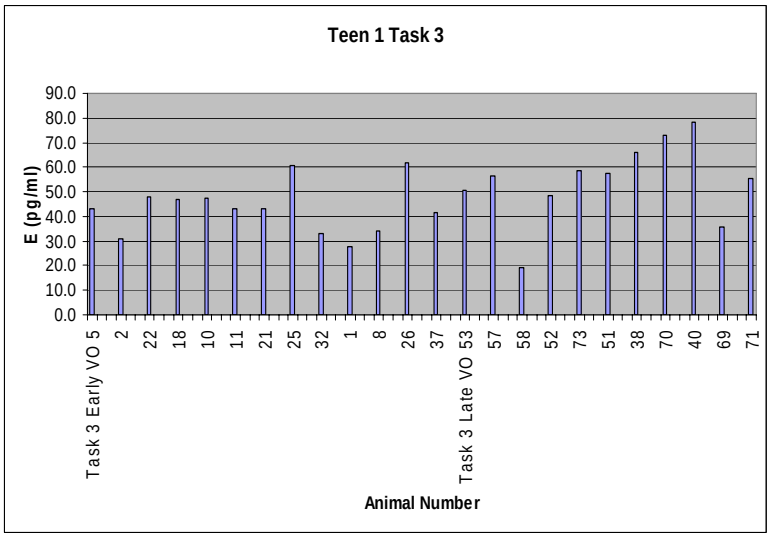

Fig. 4

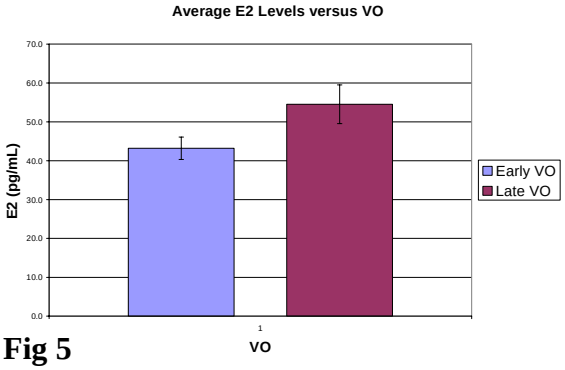

Fig 5

Figs 5& 6 Circulating estradiol levels from individual rats with early (left side of graph) and late VO (right side of graph) (**Fig 5**) and average estradiol levels per group (**Fig 6**). Late VO group average estradiol levels significantly higher than early VO group, p=0.03.

Conclusions: At all one of three stages of estrous evaluated, proestrus, estrus and diestrus, adult rats from the late VO group had higher levels of circulating estrogen than rats from the early VO group. However, only levels measured during estrus were significantly different between groups. Progesterone and IGF levels have not been determined.

Task 3: Determine whether age at VO alters the number of mammary epithelial cells expressing ERα, ERβ, PRA and PRB. Months 6-8. Rationale: An alternative mechanism by which early age menarche increases risk for breast cancer is by persistently altering the number of mammary epithelial cells expressing hormone receptors or by altering the ratio of ERα/ERβ and PRA/PRB; alterations of which are observed in many human breast cancers. The number of MEC expressing steroid receptors will be determined by IHC. For these IHC analyses, 10 rats per group were euthanized during the estrus phase of the estrous cycle (stage of cycle maximally responsive to hormonal stimulation) and mammary tissue adjacent to the lymph node chain in mammary gland #4 harvested to minimize differences in gland morphology due to proximal/distal location in the gland. Stage of estrous was confirmed by histological evaluation of cervical tissue. Mammary tissue was fixed for 24hr in 10% neutral buffered formalin, embedded in paraffin and cut into 5-µm sections.

Status: ERα and ERβ IHC staining has been completed, PRA and PRB IHC staining has not been performed.

Results: Differences in ERα or ERβ levels were not observed between the early and late VO groups. For ERα, expression levels ranged from ~5-20% positive ME cells within a group and between groups. Expression of ERβ was higher, with as many as 70% of epithelial cells staining positive, but again, differences between groups were not observed.

Task 4. Determine whether age at VO alters response of mature gland to hormone stimulation. Months 6-8

Task 4a. Determine whether age at VO alters the percent of MECs that undergo proliferation in response to hormonal stimulation. Rationale: In response to the cyclic hormonal stimulation, approximately 15% of MEC per cycle undergo a round of proliferation. One mechanism which could account for the increased risk in breast cancer risk with early menarche is that a higher proportion of MEC respond to the circulating E&P by undergoing proliferation. To address this question, the effect of VO on the proliferative index of mammary epithelium challenged with the mitogenic hormones estrogen and progesterone will be determined. At p63, 5 rats per group will be ovariectomized. One week post-ovariectomy, rats will be challenged with 5 µg 17-β-estradiol and 1.5 mg progesterone. Twelve hours post hormone injection, animals will be treated with 50 mg BrdU/kg body weight by i.p. injection and animals sacrificed two hours later. Mammary tissue, collected from gland #4 and controlled for proximal/distal location within the gland, will be fixed in methacarn, a fixative compatible for immunohistochemical detection of BrdU using anti BrdU antibody from Becton Dickinson.
Status: Alternative approach taken as described below and analyses completed.

Results: Evaluation of histological sections demonstrated that our hormone treatment protocol failed to stimulate hormone responsive tissue, such as the cervix or mammary tissue. Thus, it was not possible to determine whether early and late VO groups differed in response to hormone stimulation. Alternatively, we performed a careful proliferation analysis on mammary glands from early and late VO under endogenous hormone stimulating conditions. For this analysis, all rats were in verifiable estrus at time of tissue harvest. Two hours prior to animal sacrifice, rats were injected with 50 mg BrdU/kg body weight by i.p. injection, and mammary tissue processed for IHC detection of BrdU. For each rat evaluated, 5 random 40X objective fields were captured and converted to an 8’’x10’’ coded photo. Total number of epithelial cells per photo and number of BrdU positive cells per photo were quantitated by two independent investigators blinded to group. An average of 2000 MEC were counted per mammary gland per rat. For the early VO group, cell counts were obtained from 11 rats, and for the late VO group cell counts were obtained from 8 rats. Two rats in the late VO group were lost from study due to failure to detect BrdU positive cells in the lymph node. Rats with early VO had lower BrdU incorporation compared to rats in the late VO group, but these differences did not reach statistical significance (**Figs 7 & 8**).

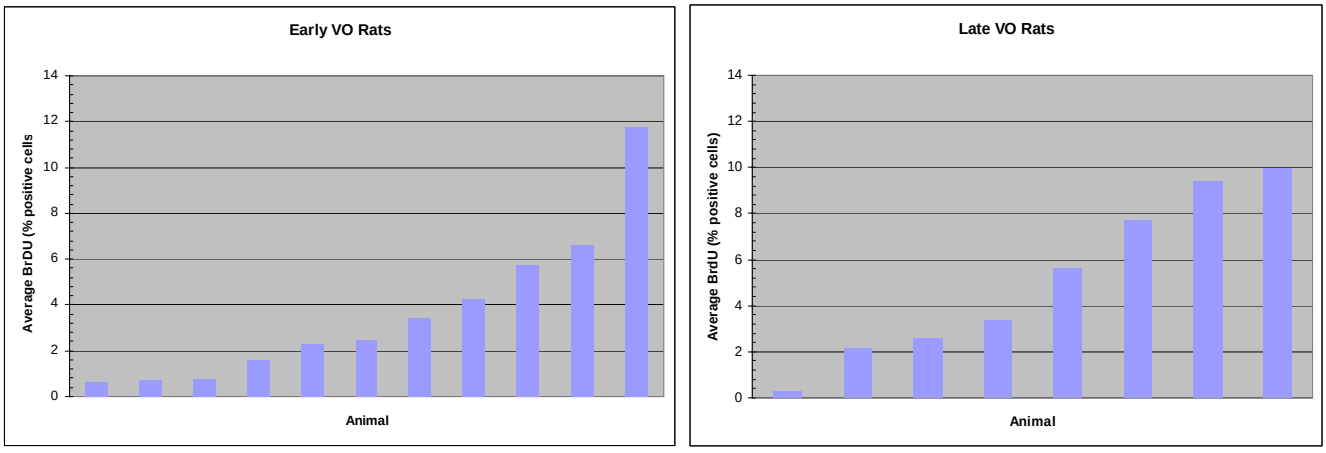

Fig 7. BrdU counts in MEC per individual animal, displayed from lowest to highest incorporation as determined by IHC. Mammary MEC BrdU incorporation counts from rats in the early VO group (left panel, n=11) and late VO group (right panel, n=8).

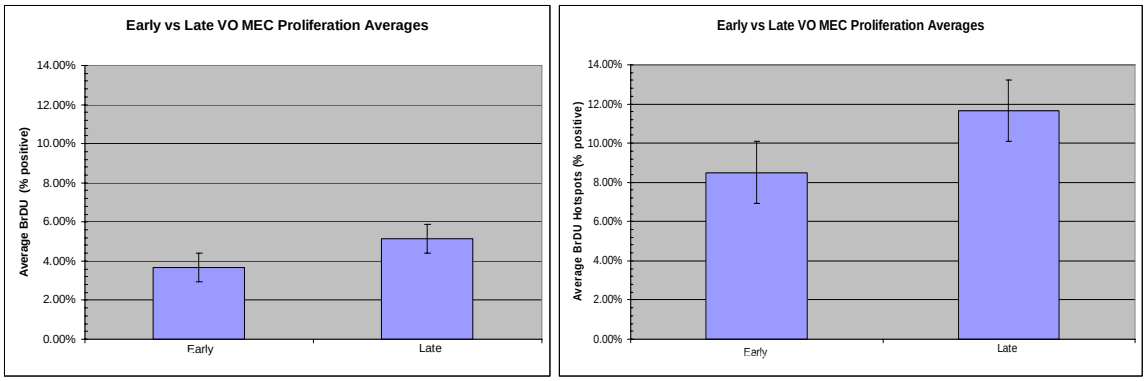

Fig 8. BrdU counts averaged per group by representative fields (left panel) and BrdU incorporation 'hot spots" (right panel. A trend for increased BrdU incorporation was observed in the late VO group compared to the early VO group, but these differences did not reach statistical significance.

Task 4b. Evaluate the effect of VO on MEC apoptotic index. Rationale: In order to maintain gland homeostasis under the conditions of repeated cyclic ovarian hormone stimulation, the number of MECs that undergo apoptotic cell death is equaled to the number that proliferate during each cycle. A reduction in the number of cells susceptible to estrous cycle-dependent cell death would be anticipated to increase risk for carcinogenesis. To address the question of whether early VO results in fewer MEC undergoing cell death, the number of apoptotic cells in the mammary glands of rats described in Task 4a will be determined. Fragmented DNA in apoptotic cells will be labeled at 3'-OH ends in situ using the TUNEL assay with digoxigenin-labeled dUTP and detected immunohistochemically using anti-digoxigenin antibody following manufacturer's protocol (R&D Systems). Percent apoptotic cells will be evaluated as described for quantization of proliferating cells described in **Task 4a**.
Status: Animal work is completed and tissues have been processed to paraffin-embedded block stage.
Results: TUNEL analysis has not yet begun.

Task 4c.
Determine whether age at VO influences mammary gland alveolar morphology.
Rationale: A correlation exists between the degree of mammary alveolar development and susceptibility to carcinogenesis. Further, exogenous progesterone increases alveologenesis and can increase risk for breast cancer in women. We will determine whether mammary gland alveolar development is altered by VO, using morphological criteria originally described by the Russos.
Status: Completed.

Results: All mammary tissue was harvested from rats in estrus stage of cycle. The inter-animal variation in mammary gland alveolar development was high within each group. The range of phenotypes observed is shown in figure 9, as well as the 1-6 grading scale utilized in our morphological assessment of alveolar development.

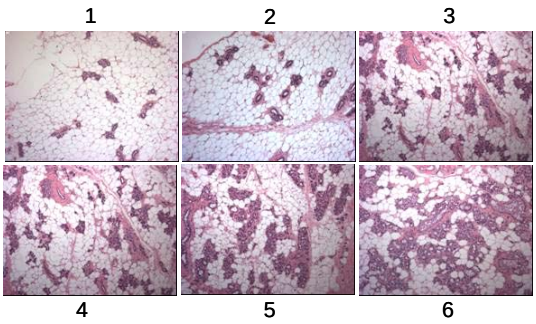

Fig 9. Morphological grading scale for alveolar development within the mammary gland. An increase in numerical value indicates an increase in alveolar density as shown.

Using the mophological scale described in Fig 9, mammary gland morphology from rats in the early and late VO groups were determined and results are shown in fig 10. Notable is the remarkably greater uniformity in gland morphology found in the late VO group, where half of rats had mammary glands with a score less than 2.0 and 83% had a score less than 3.0. This differs from the greater range shown by the early-VO group, where only 27% had a score less than 2.0 and 60% less than 3.0. Thus, a difference is apparent, but is not highly significant.

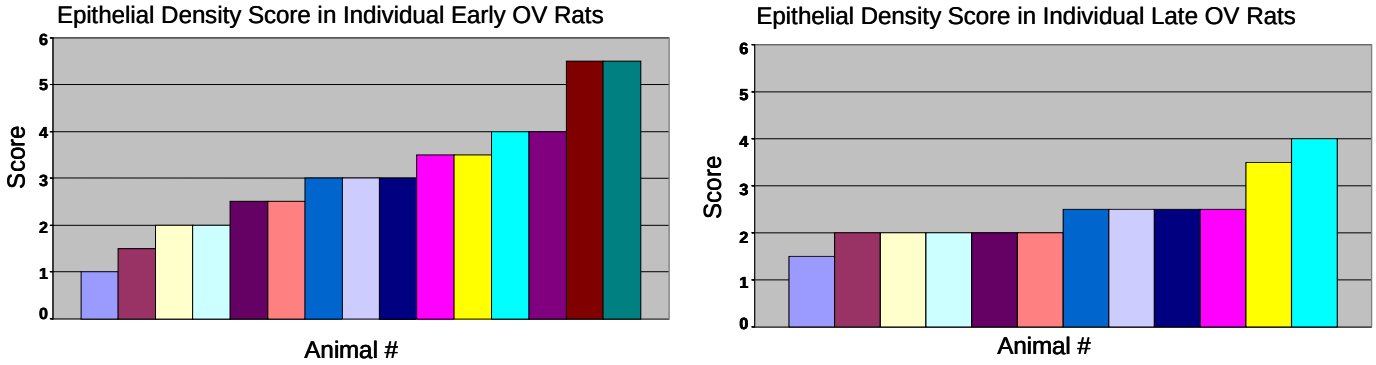

Fig 10. Morphological grading for alveolar development. Morphological score for individual rats within a group are shown arranged from lowest to highest degree of alveolar development. Early VO rats had more animals with alveolar development (right panel) than rats in the late VO group.

Task 5. Determine whether age at VO alters response of gland to carcinogenic insult.
Months 3-12

At p70, all remaining rats (40 per group) will be injected i.p. with 50 mg MNU per kg body weight and tumor incidence, latency and multiplicity followed for 6 months by twice-weekly manual palpation**.** At necropsy, tumors will be harvested, weighed and processed for histological evaluation. Only confirmed adenocarcinomas will be included in statistical analyses for tumor latency, incidence, multiplicity and tumor burden.

Status: As originally proposed, it was anticipated that this carcinogenesis study could be accomplished along with the above described tasks from a starting population of 200 rats. However, considerably fewer animals segregated into the early and late VO groups than expected, thus this arm of the study has been delayed. Our strategy was to determine whether differences between the early and late VO groups could be identified, as described in Task 2-4 prior to initiating the carcinogenesis study. Identifying biological differences in hormone signaling would justify the significant added expense of replicating the animal husbandry component of this grant. Given that significant differences in gland morphology, cell proliferation rates, estradiol levels and ERα and ERβ levels were not observed between the early and late VO groups, it was determined that a costly carcinogenesis study was not warranted.

Key Research Accomplishments:

1. Using the Sprague-Dawley female rat, we have determined that it is possible to segregate rats into those with early VO and late VO.
2. We have generated data that suggests that the relationship between body weight and age at VO is complex. Overall, the rats that had early VO were as a group heavier than the rats that had late VO. This data models the relationship observed between onset of puberty and body weight in girls. However, animals in the early VO group reached sexual maturation at a much lower body weight than rats in the late VO group. These data suggest that body weight alone does not account for entry into VO, but rather

a combination of body weight in conjunction with a 'permissive' endocrine status/profile is likely to be responsible for onset of VO.

3. We have found that rats with late VO have modestly higher circulating levels of estradiol during the estrus stage of the cycle. Whether this trend will hold up for other mammotrophic hormones (progesterone and IGF-1) remains to be determined. This result is opposite of what was hypothesized, and the significance of this observation is currently unknown.
4. Early onset of sexual maturation does not correlate with higher levels of ERα or ERβ in mammary epithelial cells.
5. BrdU incorporation in mammary epithelial cells, as a marker for cell proliferation, was modestly higher in rats with late VO compared to early, consistent with the slightly higher levels of circulating levels of estrogen observed in this group. However, the trend towards increased proliferation in the late VO group did not reach statistical significance. Further, this trend is opposite of what was anticipated.
6. Mammary glands of rats in the early VO group had more alveolar development compared to rats in the late VO group. Mammary alveolar development is primarily dependent on progesterone and prolactin. The question of whether differences in these two hormones are observed between groups is of interest.

Reportable Outcomes:

1. Provided a summer intern training opportunity for Thomas Sweed, a second year medical student at UCHSC, June 2007-August 2007.
2. Provided a summer intern training opportunity for Reema Mallick, senior at the University of Akron, Ohio, as a Colorado Cancer Center 2007 Intern, June-August, 2007.

Conclusions: The objective of this concept award was to determine whether the Sprague Dawley female rat can be used as a model to study the affect of onset of sexual maturation on breast cancer risk. Early data was encouraging, in that onset of VO in the rat correlated with body weight differences, and thus modeled human data. However, subsequent characterizations of the mammary glands from early and late onset VO groups have not identified any clear biological differences between groups. Data interpretation is confounded by wide variation between animals within the same group with respect to circulating estrogen levels, ERα and ERβ levels, mammary epithelial cell proliferation rates and mammary gland morphology. Wide inter-animal variations were observed even thought animals were carefully controlled for regularity of estrous cycling and stage of estrous at time of tissue harvest. Thus, the model does not appear to be suitable for addressing the important question of how age at sexual maturation influences mammary carcinogenesis.

References: None
Appendices: None